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ORIGINAL PAPER

The use of SR-FTIR microspectroscopy for a preliminary biochemical study of the rat hippocampal formation tissue in case of pilocarpine induced epilepsy and neutroprotection with FK-506

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Abstract. The main aim of the work was the biochemical analysis of the hippocampal formation tissue in the case of epileptic rats treated with the neuroprotective agent FK-506. Three groups of animals were compared: rats with pilocarpine induced seizures treated and non-treated with tacrolimus as well as naive controls. Synchrotron radiation Fourier transform infrared (SR-FTIR) microspectroscopy was used for the biomolecular analysis of studied samples. The measurements were carried out at SISSI beamline of ELETTRA. A Bruker IFS 66v/S interferometer coupled to a Bruker Hyperion 2000 microscope was used. The tissue samples were analyzed in transmission mode with a beam defined by a small aperture and spatial resolution steps of 10 μm which allowed us to probe the selected cross-line of the sample at cellular resolution. The obtained results enabled to compare the distributions of proteins and lipids in the three hippocampal cellular layers, i.e. in molecular, multiform and granular layers. For epileptic animals both treated and non-treated with FK-506, the tendency for increase of the ratio of the absorbance at around 1548 and 1658 cm^{-1} (amide II/amide I ratio) was observed, however only for the multiform layer these changes were statistically significant. Similar relation was noticed in case of the ratio of the absorbance at around 1631 and 1658 cm^{-1}. The mentioned results may suggest conformational changes of proteins in the direction of β-sheet secondary structure. Additionally, a statistically significant increase in the lipid massif and a decrease of the ratio of absorbance at around 2921 and 2958 cm^{-1} were observed for epileptic animals treated with tacrolimus comparing to the control group.

Key words: pilocarpine-induced epilepsy • FK-506 (tacrolimus) • SR-FTIR microspectroscopy • biochemical analysis

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Introduction

Epilepsy is one of the most common serious neurological diseases. Nevertheless, the epileptogenesis is still not known and has been a subject of intensive investigations in which animal models are used. Presented results are based on the most often used experimental model of temporal lobe epilepsy pilocarpine-induced seizures [22, 26]. It is known that epileptic seizures strongly modify internal conditions within the nervous tissue. There are many changes in neurotransmitter release, gene activation, and elemental composition [14]. In the context of biology of the epileptic processes it is needed to reveal the relationship between the elemental and biochemical changes in the tissue affected by seizures.

Beside the etiology of epilepsy animal models provide information on the action of new antiepileptic drugs. Neuroprotective factors are the agents used to minimize the pathological effects of seizures on nervous tissue. Their functions rely on increasing the ability of nerve cells to survive under pathological conditions. In the present paper an immunosuppressive drug FK-506, widely used in transplantology [1, 12, 27], was applied as neuroprotective agent.

In the present study synchrotron radiation Fourier transform infrared (SR-FTIR) microspectroscopy was used for the biomolecular analysis of studied samples. The principles underlying infrared spectroscopy is the resonant absorption of infrared light through vibrating molecules. The absorption spectrum recorded in mid-infrared region can be treated as a fingerprint of chemical compounds. The combination of infrared spectroscopy and optical microscopy as well as the use of synchrotron source of infrared allow to achieve high spectral resolution at subcellular level [4, 18].

The usefulness of FTIR microspectroscopy has been revealed by several investigations upon anomalies occurring in the disordered nervous tissue. Among others, the method was successfully applied for the analysis of protein conformational changes in case of Alzheimer's disease and prion diseases [2, 3, 13, 15, 17]. It was also used to monitor the level of exercise induced oxidative stress [19, 21].

Materials and methods

Induction of epilepsy and FK-506 administration

The subject of investigations was the rat hippocampal formation tissue. The samples were taken from the adult 60-day-old male Wistar rats obtained from an animal colony of the Institute of Pediatrics, Collegium Medicum, Jagiellonian University (Kraków, Poland). In order to induce seizures in rats, a single intraperitoneal injection of pilocarpine (300 mg/kg, Sigma P6503) was done between 9 and 10 a.m. to avoid circadian changes in seizure vulnerability. Afterwards, the animals were subjected to continuous behavioral observations during a 6 h period following the pilocarpine injection. The aim of the study was the biochemical analysis of the brain tissue from epileptic animals which were treated or not with FK-506 (Prograf, Fujisawa, 2 mg/kg). The detailed procedures concerning the application of neuroprotective agent were described elsewhere [5, 6].

Sample preparation

Six hours after epilepsy induction, all animals were perfused with physiological saline solution of high analytical purity. The brains were excised, frozen and cut using a cryomicrotome into 15 μm thick sections. Such prepared tissue slices were placed on a Ultralene® foil.

Measurements

SR-FTIR microspectroscopy was used for the biomolecular analysis. The measurements were carried out at SISSI beamline of ELETTRA. A Bruker IFS 66v/S interferometer coupled to a Bruker Hyperion 2000 microscope was used. The samples were analyzed in transmission mode with a beam defined by a small aperture and spatial resolution steps of 10 μm which allowed us to probe the selected cross-lines of the tissues at cellular resolution [8]. The spectra were collected for the wavenumber range from 1000 to 4000 cm^{-1} with a spectral resolution of 4 cm^{-1} and 128 scans were coadded per spectrum. The data collection and analysis was performed with the OPUS software (version 6.5).

The measurements were done for the three animal groups, i.e. epileptic rats treated with FK-506 (N), non-treated with neuroprotective drug (E) as well as naive control animals (C). The distributions of proteins and lipids in the three hippocampal cellular layers, i.e. in molecular (mo), multiform (mu) and granular (g) were compared.

Results

For all the analyzed tissues, the vertical line scans were done along the middle part of the hippocampal formation. The data treatment was done in the following steps:

1. Identification of the absorption bands present in the spectra collected for the tissue samples.
2. Evaluation of the selected absorption bands intensity.
3. Identification of specific cellular layers within the scanned areas.
4. Calculation of the median bands intensities and ratios of intensities for each of the observed cellular layers.
5. Comparative analysis of the results obtained for the three analyzed animal groups.
6. Statistical analysis of the significance of the differences between analyzed animal groups (U Mann-Whitney test, significance level 0.1).

Figure 1 shows the location of different cellular layers for selected samples from all the analyzed groups. In Fig. 2 the exemplary spectra recorded for different hippocampal layers (mu, mo, g) from a selected epileptic sample are compared.

Infrared absorption bands are related to the particular functional groups of biomolecules [18]. The tentative assignments for the bands occurring within the analyzed wavenumber range are presented in Table 1 [8, 17, 20, 21]. In this study intensities (ratios of intensities) of the following absorption bands were used for the analysis of the distributions of biomolecules and their structural changes:

1. The ratio of the intensities of 1548 cm^{-1} (amide II) and 1657 cm^{-1} (amide I) bands and the ratio of the absorption at around 1631 and 1657 cm^{-1} were applied for the analysis of the relative secondary structure of proteins.
2. The absorption in the range of wavenumber from around 2820 to 2996 cm^{1} (lipid massif) was used for the determination of lipid distribution.
3. The ratio of the absorbance at around 2921 and 2958 cm^{-1} was used for the analysis of the structural changes of lipids.

The median values of the analyzed parameters for the three investigated animal groups are presented in Table 2. The statistical significance of the differences between medians was tested with the non-parametric U (Mann-Whitney) test at a significance level of 0.1. The results of the statistical analysis are shown in Fig. 3.

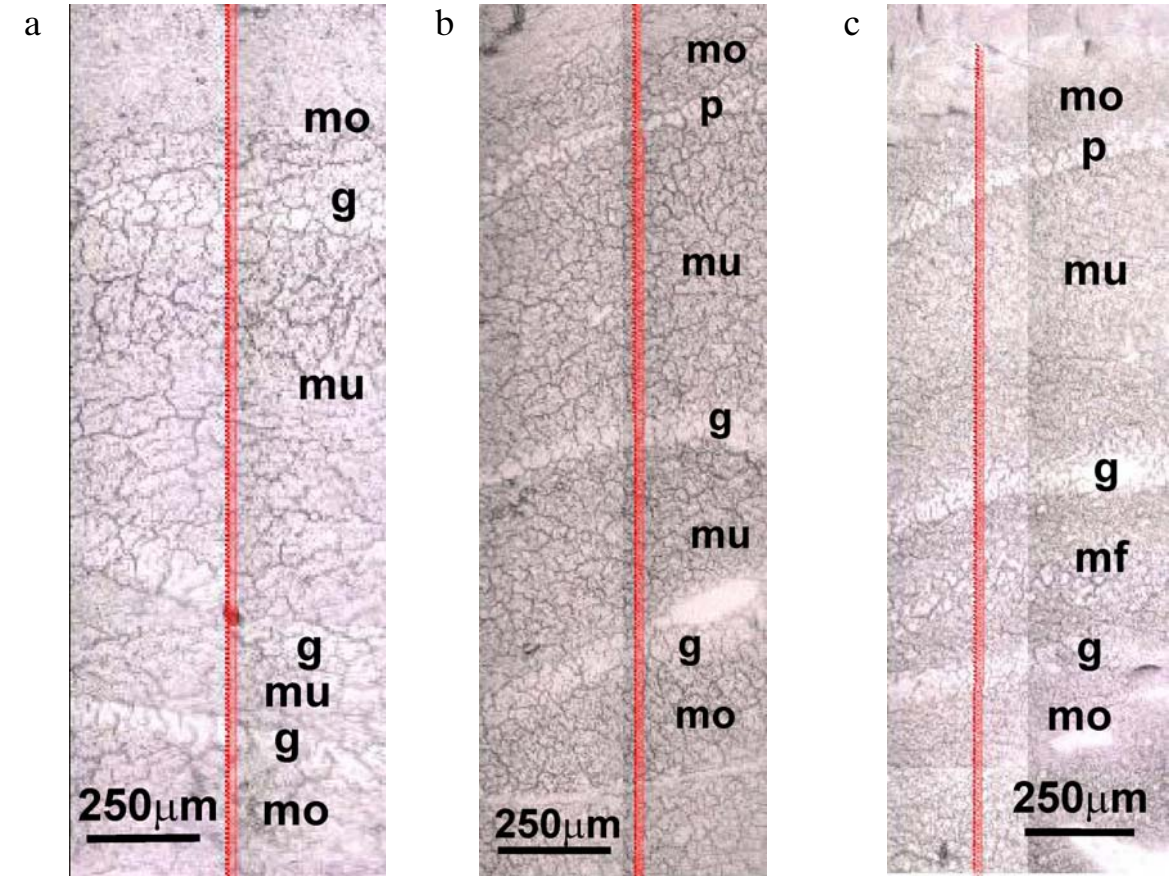

Fig. 1. Visible light microscope pictures of the cross-sections of hippocampal formations from (a) N, (b) E and (c) C groups respectively. The lines show the courses of the line scans.

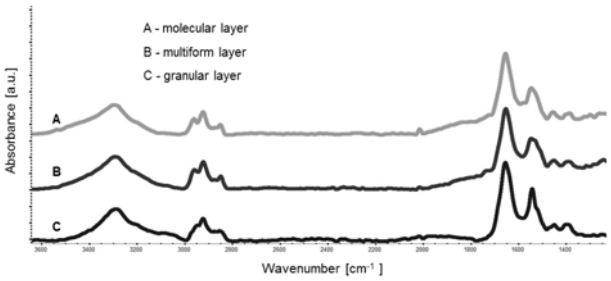

Fig. 2. Exemplary spectra recorded for different hippocampal layers (mo, mu, g) from a selected epileptic sample.

Discussion

The main aim of the study was to investigate the distributions of predominant biomolecular components of epileptic hippocampal formations taken from rats treated and non-treated with the neuroprotective agent FK-506. Moreover, structural changes of proteins and lipids were examined. The two mentioned parameters are known to be related to oxidative stress being one of the mechanisms involved in the pathogenesis and progress of neurodegenerative changes [23]. Oxidative stress is promoted by reactive oxygen species (ROS) – free radicals. Their direct monitoring is difficult due to

Table 1. Tentative band assignments for IR spectra of lipids and proteins [8, 17, 20, 21]

Frequency (cm^{-1})	Assignments
~ 3500–3200	O-H str (hydroxyl groups)
~ 3200–3300	N-H str (proteins, amide A)
~ 3080	N-H str (proteins, amide B)
~ 3020–3000	C-H str (unsaturated fatty acids, cholesterol esters)
~ 2990–2950	C-H_3 asym str (phospholipides, cholesterol esters, fatty acids)
~ 2950–2880	C-H_2 asym str (phospholipides, long chain fatty acids)
~ 2880–2860	C-H_3 sym str (phospholipides, fatty acids)
~ 2870–2830	C-H_2 sym str (phospholipides, long chain fatty acids)
~ 1739–1713	C=O str (phospholipides, cholesterol esters, glycerides)
~ 1713–1589	C=O str (proteins, lipids)
~ 1645	sphingolipides
~ 1655	amide I
~ 1589–1474	N-H bend (proteins, lipids)
~ 1545	amide II, sphingolipides
~ 1480–1430	C-H_3 bend asym, C-H_2 bend asym, C-H_3 bend sym, C-H_2 bend sym (phospholipides, fatty acids, glycerides)

Table 2. The median values of the analyzed parameters calculated for N, E and C groups

	N			E			C		
	mo	mu	g	mo	mu	g	mo	mu	g
1548/1657 cm^{-1}	0.471	0.472	0.474	0.469	0.464	0.470	0.460	0.461	0.464
1631/1657 cm^{-1}	0.685	0.691	0.706	0.713	0.705	0.684	0.645	0.656	0.650
Lipid massif	18.13	18.47	19.16	11.94	12.76	15.35	11.34	12.48	11.72
2921/2958 cm^{-1}	1.750	1.726	1.713	1.916	1.732	1.711	1.913	2.00	1.886

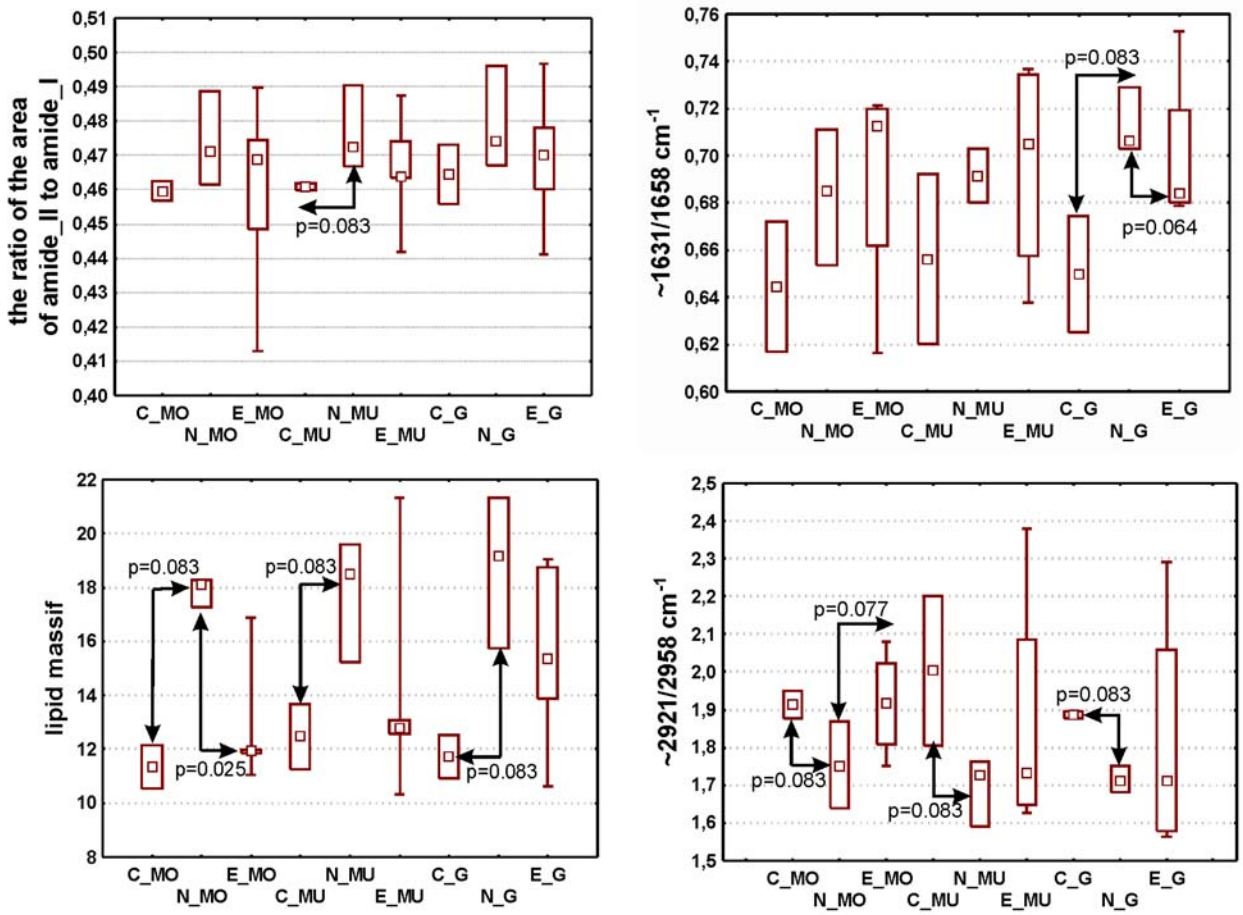

Fig. 3. The medians of selected bands intensities or ratios of intensities.

their transient and unstable nature. However, indirect markers of oxidative stress can be used and one of them is an increased of lipid peroxidation. Petibois and Deleris, in their papers from 2005 and 2006 [20, 21], showed that the ratio of intensities of CH_2 to CH_3 asymmetric stretchings provides information on mean saturation level of phospholipides being a measure of lipid peroxidation as a result of oxidation stress. Lipid peroxidation means unspecific oxidation of polyunsaturated fatty acids present in phospholipides of biological membranes. Polyunsaturated fatty acids are highly susceptible to be oxidized by ROS [28]. According to Kretlow [16] the mentioned spectra parameter may also depend on the length of the fatty acid chain.

In turn, proteins exposed to free radical action may exhibit altered primary, secondary and tertiary structure as well as may undergo spontaneous fragmentation and manifest increased proteolysis susceptibility [7, 28].

An excessive free radical formation and an increase in lipid peroxidation have been observed before in different brain areas during status epilepticus induced by pilocarpine and in the lithium-pilocarpine model [9–11, 24, 25, 28]. Most of the experiments were performed on homogenized samples using TBARS (thiobarbituric acid reactive substances) method which measures malondialdehyde present in the sample as well as malondialdehyde that is generated from certain primary and secondary lipid peroxidation products.

Conclusions

1. For both epileptic groups (N and E), the ratio of amide II to amide I tended to increase. It was observed for all of the studied cellular layers (mo, mu, g) but only for a multiform (mu) layer these changes were statistically significant.
2. Similar relation was noticed in case of the ratio of absorbance at around 1631 and 1658 cm^{-1}. In this case, statistically significant changes were observed only for granular (g) cell layer. The increased ratio of absorbance at 1631 and 1658 cm^{-1} might suggest conformational changes of proteins in the direction of β-sheet secondary structure.

3. A statistically significant increase of absorption in the region of lipid massif was noticed for N group in comparison with both E and C groups.
4. There was a statistically significant decrease in the ratio of absorbance at around 2921 and 2958 cm^{-1} for N group comparing to controls. It might suggest lipid structural changes in the first experimental group.

In light of our previous results obtained for acute phase of pilocarpine induced status epilepticus [4], the decreased ratio of the absorbance at 2921 and 2958 cm^{-1} in the epileptic group treated with FK-506 comparing to control animals suggest the efficacy of the use of FK-506 in order to reduce seizure-induced biochemical anomaly of hippocampal formation.

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